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Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*

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ABSTRACT **Mycolic acids represent a major constituent of the mycobacterial cell wall complex, which provides the first line of defense against potentially lethal environmental conditions. Slow-growing pathogenic mycobacteria such as *Mycobacterium tuberculosis* modify their mycolic acids by cyclopropanation, whereas fast-growing saprophytic species such as *Mycobacterium smegmatis* do not, suggesting that this modification may be associated with an increase in oxidative stress experienced by the slow-growing species. We have demonstrated the transformation of the distal cis double bond in the major mycolic acid of *M. smegmatis* to a *cis*-cyclopropane ring upon introduction of cosmid DNA from *M. tuberculosis*. This activity was localized to a single gene (*cma1*) encoding a protein that was 34% identical to the cyclopropane fatty acid synthase from *Escherichia coli*. Adjacent regions of the DNA sequence encode open reading frames that display homology to other fatty acid biosynthetic enzymes, indicating that some of the genes required for mycolic acid biosynthesis may be clustered in this region. *M. smegmatis* overexpressing the *cma1* gene product significantly resist killing by hydrogen peroxide, suggesting that this modification may be an important adaptation of slow-growing mycobacteria to oxidative stress.**

The World Health Organization estimates that without immediate action 30 million people will die from tuberculosis in the next decade (1). The vast majority of these deaths will occur in Third World countries, with human immunodeficiency virus-infected and immunocompromised individuals in the developed world increasingly affected (2). Although there are many clinically useful drugs, there are increasing numbers of cases involving multidrug-resistant strains. Mycolic acids are major constituents of the mycobacterial cell wall, representing up to 30% of the dried cell mass, whose biosynthesis is thought to be the target of several chemotherapeutics including isoniazid, ethionamide, and thiocarlide (3, 4). In spite of the importance of these α-alkyl, β-hydroxy fatty acids as chemotherapeutic targets, little is known regarding the chemical or genetic details of their biosynthesis and function.

The structure of one predominant member of the homologous series of α-mycolates from *Mycobacterium tuberculosis* (MTB) H37Ra is shown in Fig. 1 as structure **5** (8). MTB strains, as well as other pathogenic and slow-growing species, seem to be unique in that the major modification to the mycolic acids in each species is cyclopropanation (9). Conversion of a cis double bond into a *cis*-cyclopropane is physically an extremely conservative substitution and would not be expected to have a marked effect on the physical structure of the mycolic acid (10, 11). It has been suggested in other microbial systems that cyclopropanation of plasma membrane fatty acids may offer a protective advantage to aging cultures (12). In pathogenic mycobacteria, it is also plausible that this structural modification reflects an adaptation to their hostile intracellular environment where reactive oxygen species from the macrophage would cross-link and degrade mycolic acids with surface-exposed double bonds. In this context, it is interesting that fast-growing, saprophytic mycobacterial strains fail to cyclopropanate their mycolic acids but do produce a large amount of unsaturated mycolic acids. These studies were initiated to begin to examine potential functional roles for the various structural classes of mycolic acids produced by pathogenic mycobacteria.§

MATERIALS AND METHODS

Library Construction and Southern Blot Analysis. MTB H37Ra DNA (100 μg) was partially digested with *Sau*3A1, and fragments larger than 20 kb were ligated directly to *Bam*HI-digested pYUB18 and packaged using Gigapack Gold packaging extracts (Stratagene). Approximately 15,000 *Escherichia coli* DH5α primary transformants were pooled, and cosmid DNA was extracted and used to transform mc^2155 to kanamycin resistance. For Southern blot analysis, 1 μg of genomic DNA from each organism was digested with *Bam*HI, separated on a 1% agarose gel, and transferred and probed on GeneScreen hybridization membranes according to the manufacturer's protocol (DuPont/NEN). The probe for *cma1* was a 1033-bp PCR product spanning nucleotides 320–1353 (see Fig. 3*A*).

Purification and Screening of Mycolic Acid Methyl Esters (MAMEs). Kanamycin-resistant transformants of *Mycobacterium smegmatis* (MSMG) mc^2155 were grown in 7H9 medium with OADC (Remel, Lenexa, Kansas), 0.05% Tween 80, and kanamycin (25 μg/ml) (Sigma). MAMEs were prepared and purified as described (13). The pellets were air-dried and then redissolved in a small volume (≈50 μl) of benzene before spotting 0.5 μl onto argentation TLC plates (14).

Identification of Hybrid Mycolic Acid from MSMG. The hybrid MAME was purified by preparative argentation TLC. The keto products of the MSMG/MTB hybrid MAME resulting from treatment with chromium trioxide followed by esterification with diazomethane were prepared as described (8). The pyrrolidide derivatives of the oxidized MAME products were prepared according to the method of Andersson and Holman (15) and purified on silica gel plates using petroleum ether/diethyl ether (1:1, vol/vol). The electron-impact mass spectrum of the resultant degradation products showed characteristics ions at m/z (relative intensity) 224 (46), 252 (100), 278 (15), 281 (29), 306 (40), and 335 (10), all of which are consistent with the proposed structure assuming oxidative cleavage at the trans double bond. Permanganate/periodate cleavage of the hybrid MAME was unsuccessful, suggesting that the cis double bond was, in fact, cyclopropanated, which would render it resistant to this well-established cleavage

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Abbreviations: MAME, mycolic acid methyl ester; CMAS, cyclopropane mycolic acid synthase; ORF, open reading frame; MTB, *Mycobacterium tuberculosis*; MSMG, *Mycobacterium smegmatis*.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U27357).

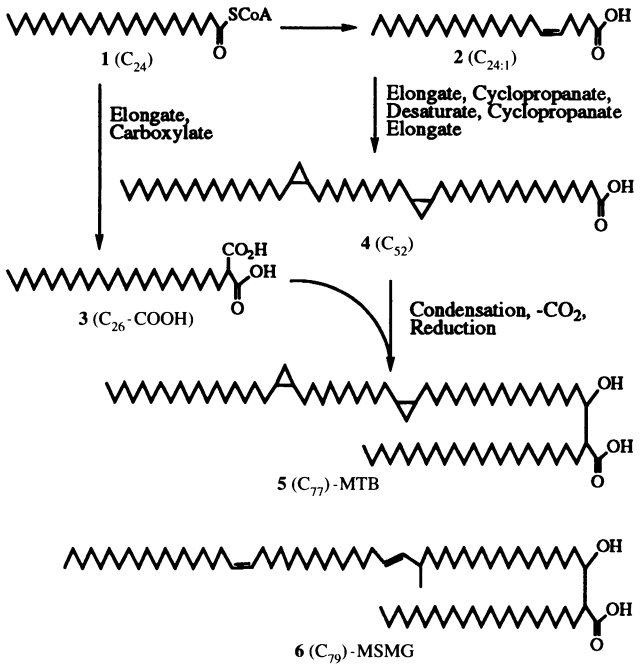

FIG. 1. Proposed biosynthetic pathway for mycolic acids in MTB H37Ra. It is proposed that mycobacterial mycolic acids are formed by a Claisen-type condensation involving a long-chain saturated C_{24} fatty acid and a C_{52} meromycolic acid (structures **3** and **4**, respectively). The shorter α-alkyl chain in this condensation (**3**) is presumably formed directly by carboxylation of an appropriately extended saturated fatty acid (5). The longer meromycolate segment is formed by chain extension of a C_{24} saturated fatty acid through a complex series of intermediates beginning with tetracosanoic acid (**1**). Tetracosanoic acid is subsequently desaturated to give $C_{24:1}$ (**2**) [the step implicated in isoniazid sensitivity (6)] and then extended to approximately C_{32} before the first cyclopropane ring is introduced. This is followed by extension, desaturation, and formation of the second cyclopropane ring to form the meromycolate **4** (7).

procedure. ^{13}C NMR (75.5 MHz, C^2HCl_3) δ 10.71 ($\underline{C}H_2$-cyclopropane), 13.91 ($\underline{C}H_3CH_2$-), 15.58 ($\underline{C}$H-cyclopropane), 20.74 ($\underline{C}H_3CHCH{=}CH$-), 22.49 ($CH_3\underline{C}H_2CH_2$-), [25.53, 27.15, 27.22, 28.52, 28.94, 29.16, 29.22, 29.50 (multiple), 30.02, 31.73, 32.38 (methylenes)], 35.52 ($\underline{C}H_2$-β-cyclopropane), 36.50 ($\underline{C}H_2$-β-olefin), 37.05 ($\underline{C}H_2$-β-OH), 50.73 ($\underline{C}H_3CH{=}CH$), 51.30 (-$C{=}O(O\underline{C}H_3)$), 72.10 [-$\underline{C}H(CH_3)CH{=}CH$-], 77.00 (-$\underline{C}$H-OH), 128.22 (-$CH_2\underline{C}H{=}CH$-), 136.27 (-$CH{=}\underline{C}H$-$CH(CH_3)$-), 176.03 [-$\underline{C}{=}O(OCH_3)$].

Hydrogen Peroxide Sensitivity Measurements. The *cma1* gene was amplified by PCR (16) and placed in-frame into pMX1, a derivative of p16R1 that has been modified by the addition at the *Kpn* I site of 200 bp of the mycobacterial *HSP60* promoter and the multiple cloning region and fusion peptide sequences of pRSETB (Invitrogen). This construct was determined to convert 25% of the total mycolates of MSMG to the cyclopropanated type by NMR. For peroxide sensitivity measurements, growth rate-matched logarithmic-phase cultures of freshly transformed mc^2155 were grown in 7H9, ADC (made without added catalases or oleic acid), 0.05% Tween 80, and hygromycin B (Calbiochem) at 50 μg/ml to early logarithmic phase ($OD_{650} \approx 0.05$). Hydrogen peroxide was added to the indicated concentration, and the cultures were incubated for 2 hr at 37°C before diluting in fresh medium and plating onto 7H11 plates.

Other Procedures. ^1H and ^{13}C NMR spectroscopy and electron-impact mass spectroscopy were performed as described (17). DNA sequence was determined simultaneously on six *Bam*HI–*Eco*RI and *Eco*RI–*Eco*RI fragments subcloned into pBluescript II (Stratagene) using the Sequenase 7-deaza-dGTP DNA sequencing kit (United States Biochemical) with synthetic universal and custom primers. In all cases, the restriction junctions were confirmed on the parent template.

RESULTS AND DISCUSSION

***cma1* Expression in MSMG Results in Production of a Hybrid Mycolic Acid.** MSMG produces a diunsaturated mycolic acid (structure **6** in Fig. 1) in which the distal cis double bond is positioned the same number of methylene units from the terminal methyl of the meromycolate chain as is the *cis*-cyclopropane of the MTB H37Ra α-mycolic acid (structure **5**) (18). MSMG mc^2155, which as been well developed as a host strain for genetic manipulations (19), was transformed with a genomic cosmid library of MTB H37Ra constructed in pYUB18, an *E. coli*–mycobacterium shuttle vector (20, 21). MAMEs were isolated from 697 kanamycin-resistant clones and examined by TLC on silica gel plates that had been treated with 5% aqueous silver nitrate. This argentation TLC process allows the selective interaction of components that have cis double bonds with immobilized silver ions while other components are unaffected in their mobility (14). The purified MAMEs from H37Ra and MSMG are clearly resolved on argentation plates but not on conventional silica plates (compare lanes 1 and 2 in Fig. 2*A Right* and *Left*). Two of these clones had modified mycolates as judged by TLC (corresponding to cosmids pYUB18-Ra_{68} and pYUB18-Ra_{189}).

To unambiguously identify this MAME, we purified this component to homogeneity from mc^2155 (pYUB18-Ra_{189}) by preparative TLC on silver plates. An examination of the 500-MHz ^1H NMR spectrum (Fig. 2*B*) clearly shows resonances assigned to the cyclopropyl ring hydrogens (δ 0.62, δ 0.54, δ 0.34) as well as olefinic resonances associated with the normal MSMG major mycolate trans double bond (δ 5.32, δ 5.22, $J = 15.3$ Hz) (22). The ^{13}C NMR spectrum and examination of fragments from chromium trioxide oxidation by mass spectrometry also support this identification. The presence of these functional groups in a single mycolate is consistent with the structure shown in Fig. 2*B*, which is composed of the normal MSMG MAME with a cyclopropanated distal double bond. These isolates showed between 14% and 19% cyclopropanation, whereas control organisms were <1% cyclopropanated in crude MAMEs, possibly due to low levels of a previously described α-smegmamycolate (23).

The two independent cosmid isolates that conferred this phenotype were isolated and restriction mapped (Fig. 3*A*). *Bam*HI fragments from the cosmid with fewer fragments (pYUB18-Ra_{189}) were cloned into pYUB18, and the cyclopropanation phenotype was conferred by a 7.2-kb *Bam*HI fragment common to both original cosmid isolates (Fig. 3*A*). Subcloning revealed that this activity was associated with a 1.51-kb *Bam*HI–*Pst* I fragment, which was sufficient to confer the ability to cyclopropanate mycolic acids on MSMG.

***cma1* Is Homologous to Cyclopropane Fatty Acid Synthase.** A total of 5.1 kb was sequenced from the original cosmids and has been deposited in GenBank (accession no. U27357). As a whole, the sequence was 66% G+C, a typical value for mycobacterial DNA (28). Mycobacteria would be expected to display a very high bias towards G or C in the third codon position in regions of DNA coding for proteins (29). The base distribution within codons was analyzed in all three potential reading frames and supports the presence of three open reading frames (ORFs) (Fig. 3*A*). The assignment of these three ORFs was also supported by the presence of the appropriate translational start and stop codons and by the distribution of rare codons.

One of these three potential ORFs was entirely encoded by the 1.5-kb *Bam*HI–*Pst* I fragment, which was sufficient to induce cyclopropanation. Analysis of this protein sequence using the BLAST program (30) revealed that it displayed significant homology to the cyclopropane fatty acid synthase of

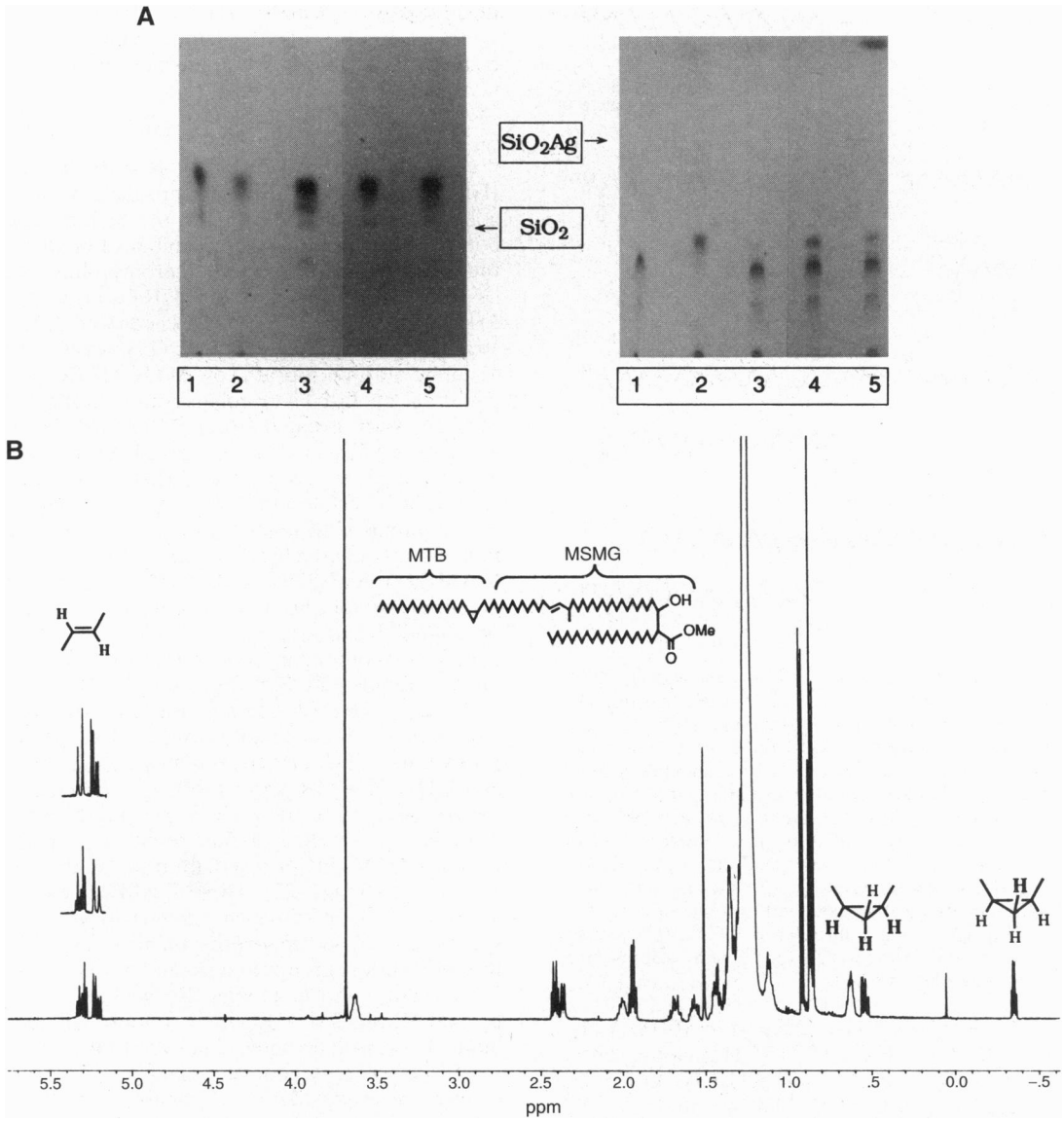

FIG. 2. The mycolic acid produced by MSMG containing an MTB DNA fragment is a chimeric structure that has features of both species. (*A*) Conventional (*Left*) and argentation (*Right*) TLC screening of MAMEs from MSMG clones containing an MTB H37Ra library. Lanes 1, MSMG mc^2155; lanes 2, MTB H37Ra; lanes 3, MSMG mc^2155 (pYUB18); lanes 4, MSMG mc^2155 (pYUB18-Ra_{68}); lanes 5, MSMG mc^2155 (pYUB18-Ra_{189}). Plates were developed twice with hexane/ethyl acetate (9:1, vol/vol) and visualized by immersion in 10% sulfuric acid in ethanol followed by charring. (*B*) ^1H NMR spectrum of the purified hybrid MAME from MSMG mc^2155 (pYUB18-Ra_{189}). Spectra were recorded at 500 MHz in C^2HCl_3. The lower offset corresponds to a decoupling experiment at δ 2.0 ppm; this decoupling also collapses the doublet at δ 0.9 ppm to a singlet, demonstrating that this olefinic proton is adjacent to a methine coupled to an α-methyl group. The upper offset corresponds to a decoupling experiment at δ 1.94 ppm and reveals that it is adjacent to the vinyl proton at δ 5.32 ppm. The coupling constant of the vinyl protons is J = 15.3 Hz, demonstrating a trans geometry. Integration of these signals proves a 1:1 relationship between the cyclopropane ring hydrogens at δ 0.7 to δ −0.4 and the vinyl protons at δ 5.25.

E. coli. This gene has been designated *cma1* (for cyclopropane mycolic acid synthase) and represents the second member of a family of genes whose protein product catalyzes the transfer of a methylene group from *S*-adenosyl-L-methionine to the double bond of a fatty acid substrate (26). On the amino acid level, the enzyme from MTB was 34% identical to the enzyme from *E. coli*, and an alignment of the two sequences is shown in Fig. 3*B*. Two areas of homology were particularly significant; the first encompasses amino acids 79–87 and corresponded to a consensus motif of (V/L)L(E/D)XGXGXG, which has been previously proposed to play a role in *S*-adenosyl-L-methionine binding (27). The second area was cysteine-290, which has been implicated in catalysis by the *E. coli* cyclopropane fatty acid synthase (26).

Interestingly, the DNA sequence corresponding to *cma1* (ORF1) also scored significantly against an unannotated cosmid deposited in GenBank as part of the *M. leprae* genome sequencing project (GenBank accession no. U00018) (25). Within the coding region, the DNA sequences were 55.2% identical while the encoded proteins were 59.5% identical (Fig. 3*B*). The significance of this homology was unclear since the sequences immediately outside the CMAS coding region were unrelated to the *M. leprae* cosmid sequences. Mycobacteria do not cyclopropanate their plasma membrane lipids (31), but there are two distinct centers in α-mycolic acids that ultimately become cyclopropanated. Thus, the lack of homology to adjacent sequences suggests that the *M. leprae* homolog encodes the cyclopropanating activity that produces the proximal *cis*-cyclopropane in structure **5** (Fig. 1).

Adjacent Sequences Encode Additional Biosynthetic Enzymes. In addition to the cyclopropanation enzyme, two additional ORFs were revealed, which had apparent homology to

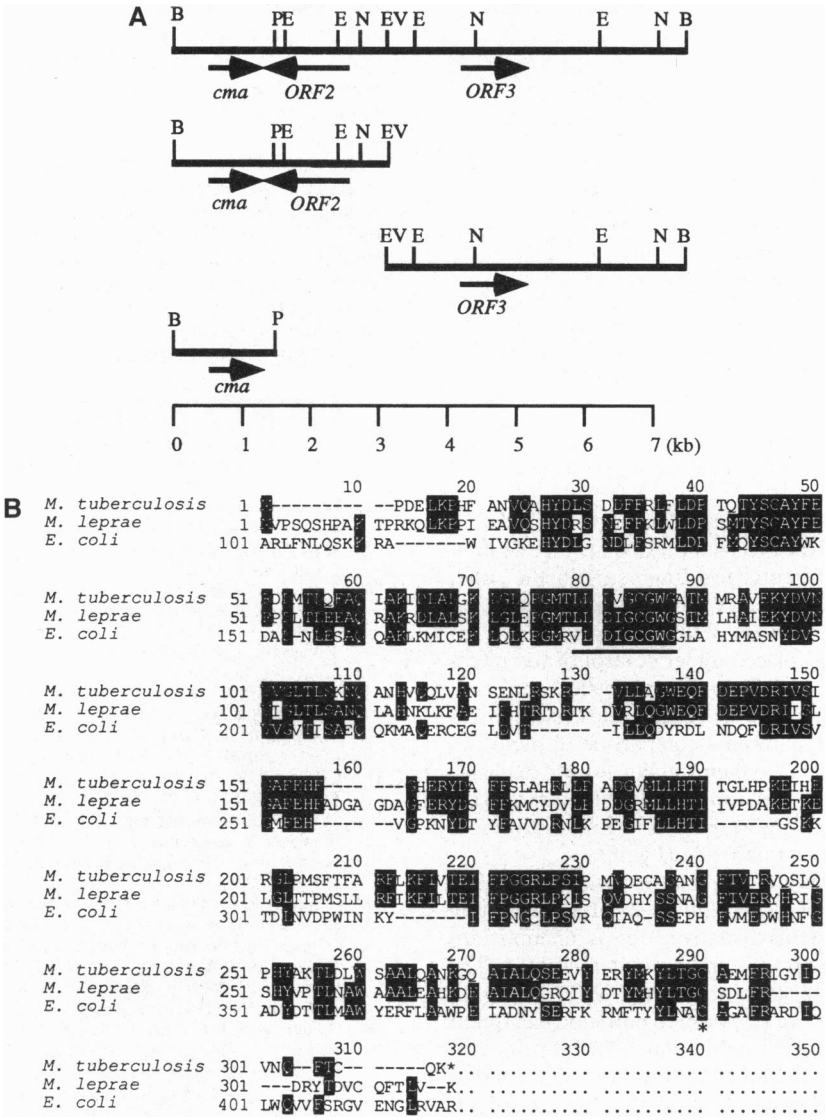

FIG. 3. An ORF encoded by this DNA is a homolog of a cyclopropane synthase from *E. coli*. (*A*) Restriction map of cosmid inserts that confer the cyclopropanation phenotype upon MSMG. The ORF contained entirely on the 1.5-kb insert was designated *cma1*. Codon preferences were deduced from the known coding sequence for the (presumably related) enzyme mycocerosic acid synthase from MTB (24). B, *Bam*HI; E, *Eco*RI; P, *Pst* I; EV, *Eco*RV; N, *Not* I. (*B*) Sequence alignment of the MTB cyclopropane mycolic acid synthase (CMAS) with an unannotated homologous sequence from the *Mycobacterium leprae* genome sequencing project (25) and the *E. coli* cyclopropane fatty acid synthase (26). The underlined portion (from amino acid 79 to amino acid 87) represents the putatative *S*-adenosyl-L-methionine binding domain (27). The asterisk highlights cysteine-290, which has been implicated in catalysis by the *E. coli* enzyme. The sequences were aligned with a Higgins multiple alignment algorithm using MACDNASIS PRO (version 3.0) (Hitachi) using a gap penalty of 100 and a window size of 5.

enzymes that may be involved in mycolic acid metabolism. The protein encoded by ORF2 was most homologous (30% identity over 188 amino acids) to ActIII, a β-ketoacyl reductase from *Streptomyces cinnamonensis*, which is involved in chain elongation in polyketide biosynthesis (32). This protein has all the strictly conserved residues to be considered a member of the family of short-chain alcohol dehydrogenases (including ActIII) that have in common a redox transformation on a fatty acid or polyketide substrate involving a nicotinamide cofactor (33). Sequence conservation, secondary structural predictions, and predicted hydrophilicity indices all supported the presence of a βαβ Rossman fold at amino acids 69–100, which has been associated with NAD(P)(H) binding (34).

The protein encoded by ORF3 was most related (35% identical over 278 amino acids) to a trifunctional hydratase/dehydrogenase/epimerase from *Candida tropicalis*, which is associated with peroxisomal degradation of fatty acids and related metabolites (35). This homology lies entirely within the C terminus, which has been implicated in the hydratase reaction and transforms a double bond into a secondary hydroxyl group. This enzyme activity in mycobacteria may represent either part of the biosynthetic system for oxygenated mycolates or a degradative system for these lipids.

***cma1* May Represent an Adaptation of Slow-Growing, Pathogenic Mycobacteria to Oxidative Stress.** To confirm that *cma1* was present in H37Ra as well as selected other pathogenic mycobacteria, a Southern blot of *Bam*HI-digested genomic DNA was probed (Fig. 4). A probe to *cma1* hybridized to a 7.2-kb fragment in all three MTB strains, confirming that this locus was conserved in both pathogenic strains of MTB. *M. marinum* and *M. avium* each possessed several fragments with significant homology to *cma1*. As expected, *cma1* had no apparent homolog in MSMG, which does not cyclopropanate its mycolic acids.

inhA has recently been described as a gene involved in resistance to isoniazid isolated from MSMG, which has been

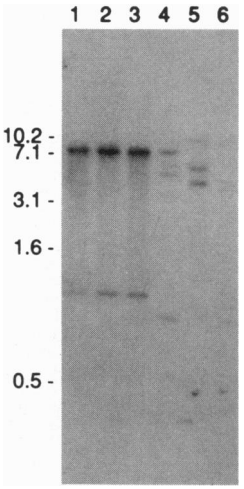

FIG. 4. Distribution of *cma1*. Southern blot of *Bam*HI-digested genomic DNA with a 1.0-kb probe corresponding to *cma1*. The lanes correspond to 1 μg of DNA from MTB H37Ra (lane 1), MTB H37Rv (lane 2), MTB Erdman (lane 3), *Mycobacterium marinum* (lane 4), *Mycobacterium avium* serovar 4 (lane 5), and MSMG mc^2155 (lane 6). Molecular size standards are shown at the left in kb.

implicated in mycolic acid biosynthesis (3). *inhA* hybridized to a 3.2-kb *Bam*HI fragment in the genomes of all three tuberculosis strains and to different-sized fragments in the other species (data not shown). Neither pYUB18-Ra_{68} nor pYUB18-Ra_{189} contain a similarly sized *Bam*HI fragment.

When the *cma1* gene was placed under control of the mycobacterial *HSP60* promoter, ≈25% of the mycolates were converted to this form (data not shown). This level of cyclopropanation at the distal position allowed a comparison of these two membrane systems with respect to their stability toward oxidizing conditions *in vitro*. Compared to plasmid-containing controls, the CMAS overexpressing clone proved to be significantly more resistant to killing by hydrogen peroxide (Fig. 5).

Hydrogen peroxide sensitivity has been shown to be directly correlated with virulence of MTB clinical isolates, suggesting that the ability to withstand this oxidative stress is an important *in vivo* determinant of persistence and pathogenicity (36, 37). Further, it has recently been shown that MTB lacks an OxyR-like inducible protective response to peroxide treatment and is susceptible to killing by millimolar concentrations of peroxide (38). The lack of an inducible response suggests that the organism may have instead adopted the strategy of increasing the intrinsic resistance of active oxygen-sensitive cellular structures. In this context, cyclopropanation of mycolic acids may be one example of such an adaptation to oxidative stress and may have a direct impact on their ability to both survive an initial respiratory burst and persist in macrophages.

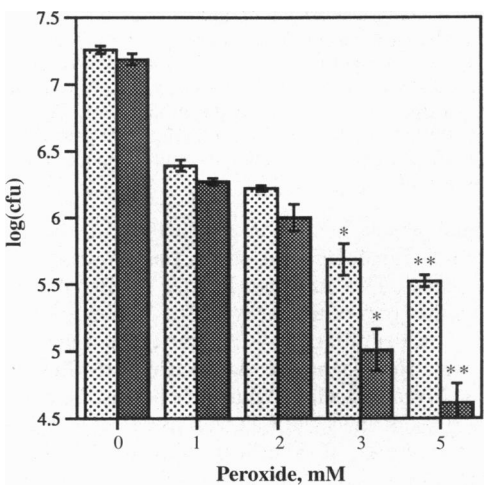

FIG. 5. CMAS overexpression increases the resistance of MSMG to killing by hydrogen peroxide. CMAS was overexpressed as a fusion protein from the mycobacterial *HSP60* promoter. This strain was then treated with hydrogen peroxide for 2 hr before dilution and plating. Light bars represent mc^2155 containing pMX1 (*cma1*), whereas dark bars represent mc^2155 containing pMX1 alone. *, Difference is statistically significant at $P = 0.004$; **, difference is statistically significant at $P = 0.0005$. cfu, colony-forming units.

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